



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/08202 <b>(22) International Filing Date:</b> 1 October 1992 (01.10.92)  <b>(30) Priority data:</b> 768,053 1 October 1991 (01.10.91) US  <b>(71) Applicant:</b> THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICE [US/US]; National Institutes of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892 (US).  <b>(72) Inventors:</b> KONIG, Monika ; 3044 R Street, N.W., Washington, DC 20007 (US). MARSH, Jon ; 8502 Jefferson Street, Bethesda, MD 20817 (US). MAHAN, Lawrence, C. ; 8016 Cindy Lane, Bethesda, MD 20817 (US). BROWNSTEIN, Michael, J. ; 6515 Old Farm Lane, Rockville, MD 20852 (US). FINK, J., Stephen ; 6 Trinity Court, Wellesley, MA 02181 (US).		<b>(74) Agents:</b> SCOTT, Watson, T. et al.; Cushman, Darby & Cushman, 1100 New York Avenue, N.W., Washington, DC 20005 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD OF IDENTIFYING LIGANDS AND ANTAGONISTS OF LIGANDS  <b>(57) Abstract</b>  The present invention relates, in general, to a method of identifying ligands and antagonists of ligands. In particular, the present invention relates to a method of identifying ligands and antagonists of ligands which bind to cloned G <sub>s</sub> - or G <sub>i</sub> -coupled receptors. The present invention also relates to a cell that comprises a recombinant cyclic AMP sensitive reporter construct.		

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**A METHOD OF IDENTIFYING LIGANDS  
AND ANTAGONISTS OF LIGANDS**

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

5           The present invention relates, in  
general, to a method of identifying ligands and  
antagonists of ligands. In particular, the present  
invention relates to a method of identifying  
ligands and antagonists of ligands which bind to  
10       cloned G,- or G,-coupled receptors. The present  
invention also relates to a cell comprising a  
recombinant cyclic AMP sensitive reporter  
construct.

**Background Information**

15           In the last several years, numerous cDNAs  
or genes for hormones and neurotransmitters that  
couple to G-proteins have been cloned. With only  
two exceptions - the receptors for insulin-like  
growth factor II and glutamic acid - these G-  
20       coupled receptors are rather similar, and they are  
thought to comprise a large "superfamily" of  
proteins. By analogy to bacterial rhodopsin, the  
members of this superfamily are thought to contain  
seven membrane-spanning domains and have a number  
25       of highly conserved amino acids. As a  
consequence, oligonucleotide probes directed at  
shared domains of cloned receptors have been used  
at low stringency to screen cDNA libraries for  
novel receptor candidates. More recently, primers  
30       directed at conserved domains of these receptors  
have been used in the polymerase chain reaction to  
clone additional candidates. The cDNAs obtained  
as described above, which encode "orphan  
receptors" for which the endogenous ligand is not

known, must be expressed and the ligands which the receptors bind must be identified. This is not as straightforward as it seems at first glance. Screening with radiolabeled ligands is expensive. It requires many (unstable) ligands and large amounts of transfected cells. Functional screens, on the other hand, are hampered by the fact that one cannot guess from the structure of a receptor which second messenger system it activates. Some G-protein coupled receptors activate phospholipase C or phospholipase A, and increase inositol phosphates and arachidonic acid, respectively. Others activate (G<sub>i</sub>-coupled) or inhibit the activation (G<sub>s</sub>-coupled) of adenylate cyclase, increasing or decreasing the level of cyclic AMP in cells. The present invention provides simple and rapid methods for discovering the agonists which act on orphan G<sub>i</sub>- or G<sub>s</sub>-coupled receptors.

The assay that has been developed employs a novel mouse L cell line, LVIP2.OZc. These L cells contain a stably integrated fusion gene pLVIP2.OZ plasmid (Riabowol, K.T. et al., (1988) Nature 336:83-86), consisting of the Escherichia coli lac Z gene under the transcriptional control of 2kb fragment derived from the vasoactive intestinal polypeptide (VIP) gene. This DNA segment includes the VIP promoter and a cyclic AMP responsive enhancer element (CRE). Forskolin increases cellular cyclic AMP levels and thus can be used to induce the  $\beta$ -galactosidase enzyme in the LVIP2.OZc. reporter cells. After the cells are lysed, the enzyme activity can be detected by addition of a chromogenic substrate, o-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG). The product of the reaction, o-nitrophenol, is yellow in color and can be seen with the naked eye or measured spectrophotometrically at a wavelength of 405nm.

The assay can be performed in 96 well tissue culture plates and a commercially available plate reader can be used to measure the levels of  $\alpha$ -nitrophenol and record the results.

5 Transfection of cells with a putative G<sub>i</sub>-coupled receptor allows one to induce the enzyme by adding the appropriate ligand. For example, cells transfected with a  $\beta$ -adrenergic receptor cDNA increase their  $\beta$ -galactosidase levels in  
10 response to isoproterenol. G<sub>i</sub>-coupled receptors, on the other hand, inhibit the activation of adenylate cyclase. In the presence of the appropriate ligands the dopamine D<sub>2</sub>, muscarinic cholinergic m<sub>1</sub>, or cannabinoid transfected  
15 receptors significantly reduce the forskolin-stimulated increase in  $\beta$ -galactosidase normally seen in LVIP2.OZc cells. Thus, LVIP.OZc cells provide a rapid, convenient and semi-automated system in which a large number of putative ligands  
20 may be screened for binding to an "orphan receptor" transiently expressed in these cells.

#### SUMMARY OF THE INVENTION

It is a general object of this invention to provide a method of identifying ligands and  
25 antagonists of ligands.

It is a specific object of this invention to provide a method of identifying ligands which bind to G-protein coupled receptors.

It is another object of this invention to  
30 provide a method of identifying an antagonist of a ligand wherein the ligand binds to a G-protein coupled receptor.

It is a further object of this invention to provide a cell that comprises a recombinant  
35 cyclic AMP sensitive reporter construct.

Further benefits and advantages of the present invention will be clear from the description that follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

5                    Figure 1. Detection of  $\beta$ -galactosidase of color development of o-nitrophenol. LVIP2.OZc cells were exposed for 8 hours to control medium (containing 0.5mM IBMX) ( $\square$ ), or control medium containing 0.5 $\mu$ M forskolin ( $\cdot$ ), or control medium  
10                   containing 1 $\mu$ M ( $\blacksquare$ ) or 10 NECA ( $\blacktriangle$ ). After cell lysis and addition of the substrate ONPG, enzyme activity was measured as optical density at 405nm at various time intervals. Data are mean  $\pm$  S.D. taken from one experiment that is representative  
15                   of several.

                  Figure 2. Induction of  $\beta$ -galactosidase as a response to length of ligand exposure. LVIP2.OZc cells were incubated for 1, 2, 4, 6, 8, 12, 15 and 18 hours in control medium (containing  
20                   0.5mM IBMX) ( $\square$ ), or control medium containing 0.5 $\mu$ M forskolin ( $\cdot$ ), or control medium containing 1 $\mu$ M NECA ( $\blacksquare$ ), or control medium containing 1 $\mu$ M PGE, ( $\blacktriangle$ ). Data are mean  $\pm$  S.D. from one representative experiment.

25                   Figure 3. Induction of  $\beta$ -galactosidase as a response to different doses of ligands. LVIP2.OZc cells were treated for 8 hours with control medium (containing 0.5mM IBMX), and 0.1 or 0.2 or 0.5 or 1 $\mu$ M forskolin ( $\cdot$ ), control medium  
30                   and 0.1 or 0.5 or 1 or 10 $\mu$ M NECA ( $\blacksquare$ ), control medium containing 0.1 or 0.5 or 1 or 10 $\mu$ M PGE, ( $\blacktriangle$ ). Data are mean  $\pm$  S.D. from a representative experiment.

Figur 4. A) Induction of  $\beta$ -galactosidase as a dose-response for  $\beta$ -adrenergic receptor DNA transfected LVIP2.OZc cells to isoproterenol. Cells were incubated for 8 hours with medium containing 0.5mM IBMX, 100 $\mu$ M ascorbic acid and  $5 \times 10^{-12}$  to  $10^{-4}$  M isoproterenol. Data are mean  $\pm$  S.D. from a representative experiment. B) Induction of  $\beta$ -galactosidase as a time-response for  $\beta$ -adrenergic receptor DNA transfected LVIP2.OZc cells to isoproterenol. Cells were exposed for 2, 4, 6, 8, 12 and 18 hours to medium containing 0.5mM IBMX, 100 $\mu$ M ascorbic acid  $\pm$  1 $\mu$ M isoproterenol. Data are mean  $\pm$  S.D. from a representative experiment.

Figure 5. Inhibition of forskolin-mediated induction of  $\beta$ -galactosidase by aldosterone secretion inhibitory factor during different exposure times. LVIP2.OZc cells were exposed for 1, 2, 4, 6, 8, 15, or 18 hours to control medium (containing 0.5mM IBMX and 20ug/ml Bacitracin) ( $\square$ ), or control medium containing 0.5 $\mu$ M forskolin + 1  $\mu$ M ASIF (O), or - ASIF ( $\cdot$ ). Data are mean  $\pm$  S.D. from a representative experiment.

Figure 6. A) Inhibition of forskolin-mediated induction of  $\beta$ -galactosidase in dopamine receptor DNA transfected LVIP2.OZc cells as a dose-response to different concentrations of dopamine or quinpirole. Cells were exposed for 6 hours to medium containing 0.5mM IBMX and 100 $\mu$ M ascorbic acid and 0.5 $\mu$ M forskolin  $\pm$  1 or 10 $\mu$ M dopamine hydrochloride or 1, 10, 100 $\mu$ M quinpirole hydrochloride. Data are mean  $\pm$  S.D. from one representative experiment. B) Inhibition of forskolin-mediated induction of  $\beta$ -galactosidase in

cannabinoid receptor DNA transfected LVIP2.OZc cells by CP55940. Cells were exposed for 6 hours to medium containing 0.5mM IBMX, 0.5mM BSA and 0.5μM forskolin ± 1μM CP55940. Data are mean ± S.D. from representative experiment. C) Inhibition for forskolin-mediated induction of β-galactosidase in human muscarinic acetylcholine (hm<sub>2</sub>) receptor DNA transfected LVIP2.OZc cells by carbachol. Cells were exposed for 6 hours to medium containing 0.5mM IBMX and 0.5μM forskolin ± mM carbachol. Data are mean ± S.D. from one representative experiment.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a fast, simple method to identify (1) ligands which bind to a G-protein coupled receptor and (2) antagonists of these ligands.

In one embodiment, the method comprises: i) expressing a G<sub>i</sub>-coupled receptor gene in a cell wherein the cell contains a cyclic AMP sensitive reporter construct; ii) adding a ligand to the cell; and iii) assaying for the amount of cyclic AMP.

In general, the construct comprises a cyclic AMP responsive regulatory element operably linked to a reporter gene. In a preferred embodiment, the cyclic AMP sensitive reporter construct comprises a cyclic AMP responsive enhancer element operably linked to a promoter and a reporter gene. Suitable cyclic AMP responsive enhancer (CRE) elements are well known in the art. They include but are not limited to the CRE located on the 2 kb DNA fragment from the vasoactive intestinal polypeptide (VIP) gene. Suitable promoters are also well known in the art.



They include but are not limited to the VIP  
promoter described herein. Further, suitable  
reporter genes are also well known in the art.  
They include but are not limited to: the Lac Z  
5 gene and the luciferase gene. Specifically, the  
reporter construct used may be pLVIP2.OZ.

The G-coupled receptor gene expressed in  
the cell may be stably incorporated into the  
genome of the cell, may be transiently transfected  
10 into the cell, or may be endogenous to the cell.  
Examples of G-coupled receptors include: the human  
 $\beta_1$ -adrenergic receptor; the human  $m_1$  muscarinic  
acetylcholine receptor; the rat cannabinoid  
receptor; the dopamine  $D_1$  receptor; VIP; secretin;  
15 vasopressin; oxytocin; serotonin;  $\alpha$ -adrenergic;  
metabolic glutamate; and IL-8 receptors.

Suitable host cells, if appropriate  
vectors are used, include both lower eucaryotes  
(for example, yeast) and higher eucaryotes (for  
20 example, mammalian cells - specifically, mouse  
cells). Preferably, the assay uses a mouse L cell  
line (for example - LVIP2.OZc, ATCC accession no.  
CRL 10871) which contains a cyclic AMP responsive  
reporter construct (for example - pLVIP2.OZ).

The methods of assaying for the  
expression of the reporter genes are well known in  
the art. In a preferred embodiment, the assaying  
step comprises adding a chromogenic substrate and  
assaying for a change in that substrate. One  
30 preferred chromogenic compound is o-nitrophenyl  $\beta$ -  
D-galactopyranoside.

In a further embodiment, the present  
invention relates to a method of identifying an  
antagonist of a ligand wherein the ligand binds to  
35 a G-protein coupled receptor. The method  
comprises: i) expressing a G-coupled receptor gene  
or cDNA in a cell wherein the cell contains a

cyclic AMP sensitive reporter construct; ii) adding a ligand and antagonist to the cell; and iii) assaying for the amount of cyclic AMP. The cyclic AMP reporters, reporter genes, constructs, and cells described-above can be used to identify antagonists.

In a further embodiment, the present invention relates to a method of identifying a ligand that binds to a receptor which inhibits stimulation of adenylyl cyclase. The method comprises: i) expressing a  $G_i$ -coupled receptor gene of cDNA in a cell wherein the cell contains a cyclic AMP sensitive reporter construct; ii) adding forskolin and candidate ligands; and iii) assaying for the amount of cyclic AMP.

In a further embodiment, the present invention relates to a method of identifying an antagonist of a ligand that binds to a receptor which inhibits stimulation of adenylyl cyclase. The method comprises: i) expressing a  $G_i$ -coupled receptor or cDNA in a cell wherein the cell contains forskolin and a specific ligand and candidate antagonists; and iii) assaying for the amount of cyclic AMP, looking for the inhibition of agonist induced reduction of forskolin activity.

The method described herein can be used to screen large numbers of ligands: for example, as many as 22 ligands can be analyzed in quadruplicate in a single 96 well plate. The stimulation or inhibition of stimulation is easy to recognize, and positive results can quickly be confirmed.

The present invention is described in further detail in the following non-limiting Examples.

### EXAMPLES

The following protocols and experimental details are referenced in the Examples that follow:

- 5 **Materials.** Unless otherwise indicated, chemicals were obtained from SIGMA, culture media from Whittaker Bioproducts, ASIF (Aldosterone Secretion Inhibitory Factor) [Bovine] was purchased from PENINSULA LABORATORIES, ONPG (o-NITROPHENYL- $\beta$ -D-galactopyranoside) was obtained from RESEARCH
- 10 ORGANICS, (-)-Propanolol hydrochloride, Dopamine hydrochloride, (-)-Quinpirole hydrochloride were obtained from RBI, CP55940 was generously provided by PFIZER, INC.
- 15 **Stable cell line and cell culture.** Mouse Ltk-cells ( $3 \times 10^6$  per 10cm dish) were transfected using the calcium phosphate co-precipitation method with the pVIP2.OZ plasmid (20  $\mu$ g) containing 2 kb of 5'-flanking sequences from the human VIP gene
- 20 fused to the E. coli lac Z gene (Riabowol et al. (1988) Nature 336:83-86). Co-transfection with 4  $\mu$ g phyg (Sugden et al. (1985) Mol. Cell. Biol. 5:410), a plasmid encoding hygromycin B phosphotransferase, and selection in hygromycin
- 25 provided a dominant selectable marker. Hygromycin-resistant Ltk<sup>-</sup> clones were screened for induction of  $\beta$ -galactosidase after addition of forskolin (10  $\mu$ M) and IBMX (0.5  $\mu$ M). One clone, LVIP2.OZc, was chosen for use in these
- 30 experiments. LVIP.OZc cells, stably transfected with the pVIP2.OZ plasmid were maintained in Dulbecco modified Eagle medium (DMEM), supplemented with 2.5% fetal calf serum, 7.5% newborn calf serum, 25 mg/ml hygromycin,
- 35 penicillin and streptomycin in a 7% CO<sub>2</sub>, 37° Forma

incubator. Cells were trypsinized twice with 1 ml 0.5% Trypsin and .53 mM EDTA.4Na /10 cm dish and split at a ratio of 1:10 and grown in 10 ml medium over several months. The phenotype of the cells seems stable.

Plasmid DNA. Plasmid DNA was prepared by the lysozyme-Triton procedure (Katz, L. et al., (1973) J. Bacteriol. 114:577-591) as modified for the transfection protocol of Chen and Okayama ((1987) Mol. Cell. Biol. 7:2745-2752). The following receptor cDNAs were introduced into the pcD plasmid (Okayama, H. et al., (1983) Mol. Cell. Biol. 3:280-289): the human  $\beta_2$ -adrenergic receptor (Kobilka, B.K. et al., (1987) PNAS 84:46-50), the human  $m_2$  muscarinic acetylcholine receptor (Bonner, T.I. et al., (1987) Science 237:527-531), the rat cannabinoid receptor (Matsuda, L.A. et al., (1990) Nature 346:561-564). The dopamine  $D_2$  receptor (Monsma, F.J. et al., (1989) Nature 342:926-929) was subcloned into the pRC/RSV vector (INVITROGEN, eukaryotic vector).

Transfection of cells. Cells were transfected by the calcium phosphate transfection protocol of Chen and Okayama. For each plasmid DNA preparation, the amount of DNA required for formation of an optimum calcium phosphate-DNA precipitate varies; thus, a dose response study is desirable. To set up conditions to assay G<sub>i</sub>-coupled receptors, cells were transfected with various amounts (5 to 30  $\mu$ g) of pcD plasmid DNA containing the human  $\beta_2$ -adrenergic receptor cDNA/10-cm plate. If smaller or larger dishes were used, the amount of DNA was adjusted proportionally. The precipitate formed with 5  $\mu$ g of DNA usually was very coarse; with 25 to 30  $\mu$ g of

DNA the precipitate was usually very fine when observed with a microscope at low magnification (40x). Eight to 20 µg gave a punctuate precipitate which seems to be taken up by the cells efficiently. Cells were trypsinized 48 hours after transfection, pelleted, resuspended in fresh medium, seeded into 96 well microtiter plates (flat bottom) at  $5-10 \times 10^4$  cells/well in 100 µl medium, and incubated for an additional 24 hours. Then 100 µl of medium containing isoproterenol (2 µM), ascorbic acid (200 µM) and isobutyl methyl xanthine (1 mM) (IBMX, a phosphodiesterase inhibitor) was added to each well. Adding medium rather than replacing the medium in each well gave more reproducible results. One hundred microliters of medium containing IBMX/ascorbic acid or forskolin (1 µM)/IBMX/ascorbic acid were added to control wells. To establish conditions to assay G<sub>i</sub>-coupled receptors, cells were transfected with the dopamine D<sub>2</sub> plasmid DNA as described above, seeded into 96 well microtiter plates in 100 µl medium and the appropriate ligands in 100 µl medium were tested for the inhibition of forskolin-stimulated β-galactosidase activity. Each set of conditions was carried out in quadruplicate. After an eight hour exposure to ligand the cells were washed with 200 µl phosphate buffered saline and β-galactosidase activity was assayed. The plates can be stored at -20°C for 24-72 hours before performing the β-galactosidase assay.

β-Galactosidase microassay. β-galactosidase activity was measured with a microplate reader (Molecular Devices, Palo Alto). The assay is a modified version of the o-nitrophenyl β-D-galactoside (ONPG)-based assays of Perrin ((1963)

Ann. N. Y. Acad. Sci. 81:6349-6353) and N rt n and Coffin ((1985) Mol. Cell. Bi l. 5:281:290). The modificati ns include th m thod of c ll lysis and conditions f assay incubati n as outlin d below.

- 5 Suppressing endog nous galact sidas activity and stabilizing the E. coli enzyme are critical for the success of the assay.

All the steps in the microassay were carried out at room temperature. The assay buffer  
10 is composed of 100 mM sodium phosphate, 2mM MgSO<sub>4</sub>, 0.1mM MnCl<sub>2</sub>, pH 8.0. The adherent cells were washed with PBS, the plate drained and 25μl/well of hypotonic lysis buffer added to each well, (dilute 1 part buffer with 9 parts water). Ten  
15 minutes later 100μl of assay buffer containing 0.5% (v/v) TritonX-100 and 40mM β-mercaptoethanol were added. Care should be taken to avoid foaming. After an additional 10 minutes, 24μl of ONPG (4mg/ml in assay buffer; prepared daily) was  
20 added, and the optical density per well at 405nm was determined with a plate reader. Readings were taken through 1.5 OD at 405nm, within the linear portion of the color development. The initial rates, expressed as mOD/minute, are linearly  
25 related to β-galactosidase enzyme concentration over an enzyme concentration range of 0.1 to 100mU (one unit hydrolyzes one micromole of ONPG per minute at 25 degrees, pH 7.5).

- 30 A detailed description of the screening protocol is provided in Table 1.

**EXAMPLE 1****Detection of  $\beta$ -D-galactosidase  
in LVIP2.OZc cells**

In preliminary experiments, forskolin  
5 (5 $\mu$ M) and IBMX (0.5mM) was used to increase cyclic  
AMP levels. The cells were treated with the drugs  
for 3, 6, or 18 hours, fixed with 2% formaldehyde  
and 0.2% glutaraldehyde for 10 minutes at room  
10 temperature, and exposed to the chromogenic  
substrate, X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-  
galactoside, 0.2 mg/ml) for 2 hours at 37°C. More  
than 90% of the cells treated with drugs for 6 to  
18 hours developed a blue color. Only a few cells  
15 treated with IBMX alone turned blue. Next, the  
cells were transfected with human  $\beta_2$ -adrenergic  
receptor cDNA in the pCD expression vector.  
Seventy-two hours after the DNA was added to the  
cells, IBMX and ascorbic acid (100 $\mu$ M) plus or  
minus isoproterenol (100 $\mu$ M) were added to the  
20 medium. Ascorbic acid was added to prevent  
oxidation of isoproterenol. Six, 9 or 18 hours  
later, the cells were fixed and stained with  
0.2mg/ml X-gal. Unfortunately, the number of  
positive cells observed following treatment with  
25 isoproterenol and IBMX was much smaller than the  
number seen following treatment with forskolin and  
IBMX. It was difficult to distinguish plates of  
cells treated with isoproterenol and IBMX from  
those exposed to IBMX alone.

30 Therefore, a quantitative assay was  
developed for the cAMP-mediated increase in  $\beta$ -  
galactosidase activity. This assay was performed  
in 96 well plates after solubilization of the  
cells and incubation with the substrate ONPG.  
35 Thus, the change in optical density over a defined  
period of time was directly proportional to the

1 vel of  $\beta$ -galactosidase activity. The optical density increased linearly with time up to 1.5. The LVIP2.0Z cells were determined to contain endogenous G<sub>i</sub>-coupled receptors for PGE<sub>2</sub>, (prostaglandin E<sub>2</sub>) and adenosine. In pilot studies, untransfected cells were treated for 8 hours with IBMX in the presence or absence of NECA [5'-(N-ETHYLCARBOXAMIDO)-ADENOSINE, adenosine A<sub>1</sub> receptor agonist], or forskolin, to increase intracellular cAMP levels (Figure 1). NECA or forskolin-treated cells generated considerably higher levels of  $\beta$ -galactosidase activity than the IBMX-treated control cells. Similar results were obtained by incubations of cells with 1 or 10  $\mu$ M PGE<sub>2</sub>. The enzyme levels generated by 1  $\mu$ M NECA, 10  $\mu$ M NECA, and 0.5  $\mu$ M forskolin were determined to be 8, 30, and 48-fold higher, respectively, than in the IBMX control cells. The differences were discernible by visual examination after 3 to 6 hours.

## EXAMPLE 2

### Induction of $\beta$ -galactosidase: optimizing duration of exposure to ligands and doses of ligands.

In pilot studies, cells were exposed to IBMX in the presence or absence for forskolin (0.5  $\mu$ M), NECA (1  $\mu$ M), or prostaglandin E<sub>2</sub> (1  $\mu$ M) for 2, 4, 6, 8, 15, or 18 hours and their  $\beta$ -galactosidase activities measured. As shown in Figure 2, the excellent signal to noise ratios were obtained with drug/ligand treatments of 6 to 15 hours. Dose-response data (Figure 3) were obtained by treating cells with forskolin, NECA, or PGE<sub>2</sub> for 8 hours. Subsequent studies have shown that 6-8 hour exposures to ligand are optimal in



both the G<sub>i</sub> and G<sub>s</sub> assays. The reference, A 6-8 hour drug treatment was used; this way, the assay can be performed in one day and the signal to noise ratio is quite good. Forskolin produced its maximum effect at a 0.5  $\mu$ M concentration; 10  $\mu$ M prostaglandin E<sub>1</sub> and NECA were required to achieve maximum enzyme induction.

### EXAMPLE 3

#### Transfection of receptor cDNAs into LVIP2.OZc cells

The high efficiency transfection method of Chen and Okayama can be used to introduce plasmids containing receptor cDNAs into LVIP2.OZc cells. As previously shown, the amount of DNA used for transfection of cells is critically important: too little or too much DNA produces poor transient or stable expression of cDNAs. Five to 10  $\mu$ g of pcD plasmid containing  $\beta_1$ -adrenergic receptor cDNA were dissolved in the calcium/BES buffered saline transfection solution and added to the 10ml of tissue culture medium in 10 cm plates of cells. This amount of plasmid gave a punctuate precipitate which could readily be seen at 40x magnification and which gave the best induction of  $\beta$ -galactosidase in response to 1  $\mu$ M isoproterenol. The increase in  $\beta$ -galactosidase activity observed with IBMX in the presence or absence of isoproterenol was 2 to 4 times that observed with IBMX alone. The increase induced by isoproterenol was blocked by coincubation with the antagonist propranolol (1  $\mu$ M).

EXAMPLE 4

Induction of  $\beta$ -galactosidase in cells  
transfected with  $\beta$ -adrenergic receptor cDNA:  
optimizing the dose of isoproterenol and duration  
of exposure of cells to this compound

Three days after transfection with plasmids containing the  $\beta$ -adrenergic receptor cDNA, LVIP2.OZc cells were seeded in 96 well plates and incubated with isoproterenol, ascorbic acid and IBMX for 8 hours. The concentration of isoproterenol was varied from  $10^{-12}$  to  $10^{-6}$ M, a maximal effect was seen at  $10^{-6}$ M; no further change in optical density was obtained for concentrations between  $10^{-6}$ M; no further change in optical density was obtained for concentrations between  $10^{-6}$  and  $10^{-4}$ M. The  $EC_{50}$  value for isoproterenol was determined to be 10pM (Figure 4A). LVIP2.OZc cells were transfected as above, seeded in 96 well plates and incubated with  $1\mu$ M isoproterenol, ascorbic acid and IBMX for 2, 4, 6, 8, 12 or 18 hours. The best signal to noise ratio was found after 6-8 hours exposure to drugs (Figure 4B).

EXAMPLE 5

Inhibition of forskolin-mediated induction  
of  $\beta$ -galactosidase by aldosterone secretion  
inhibitory factor (ASIF)

LVIP2.OZc cells were screened for endogenous receptors linked to  $G_i$ , which when activated would inhibit the increase in  $\beta$ -galactosidase activity mediated by treatment with forskolin. Among numerous agonists tested, only ASIF produced significant inhibition. ASIF was used to help optimize a screen for ligands which can interact with  $G_i$ -coupled receptors. Cells were

trated with IBMX, IBMX and forskolin ( $0.5\mu\text{M}$ ), r  
IBMX, forskolin, and ASIF ( $1\mu\text{M}$ ) for 1, 2, 4, 6, 8,  
15, or 18 hours. The most robust inhibition of  
forskolin by ASIF was observed between 6 and 8  
5 hours (Figure 5). Lower concentrations of  
forskolin ( $0.1$  to  $0.5\mu\text{M}$ ) should be used, however,  
to observe maximum inhibition in the assay. The  
highest dose of forskolin is not inhibited as well  
as the two lower doses, but the former may give a  
10 more reproducible level of enzyme induction.

#### EXAMPLE 6

##### Inhibition of forskolin-mediated induction of $\beta$ -galactosidase by dopamine or quinpirole following transfection 15 of cells with dopamine receptor cDNA

Cells were transfected with 5, 10, 15, or  
20  $\mu\text{g}$  of  $D_{\text{R}}\text{Rc/RSV}$ , a plasmid containing the  $D_{\text{R}}$   
receptor isoform. Ten  $\mu\text{g}$  of plasmid per 10 ml in  
a 10 cm dish gave the best looking precipitate,  
20 and aliquots of a suspension of cells from this  
dish were added to wells of a 96 well plate.  
Three days after the transfection, IBMX was added  
to all of the wells.  $\beta$ -galactosidase activity was  
elevated with forskolin ( $0.5\mu\text{M}$ ) alone or in  
25 combination with dopamine, or the  $D_1$  selective  
agonist, quinpirole, to block this increase. The  
dopaminergic agonists were added in concentrations  
of 1 to  $100\mu\text{M}$ .  $100\mu\text{M}$  ascorbic acid was added to  
the medium to prevent oxidation of the ligands  
30 (Figure 6A). Dopaminergic agonists alone had  
little or no effect on  $\beta$ -galactosidase levels.  
Significant inhibition of forskolin-stimulated  
increase of  $\beta$ -galactosidase activity was observed  
with either  $10\mu\text{M}$  dopamine or 10 or  $100\mu\text{M}$

quinpir le. As exp ct d, quinpir l was m re  
pot nt than dopamin .

Transf ctions and assays as describ d  
abov were us d succ ssfully to "diagn s " th  
5 rat cannabinoid or th human muscarinic m,  
receptor. Addition of 1 $\mu$ M agonist CP55940  
decreased forskolin stimulated activity by 15% in  
cells expressing the cannabinoid receptor (Figure  
6B). Similar decrease was observed after addition  
10 of 1mM carbachol to cells expressing the  
muscarinic acetylcholine receptor (Figure 6C).

Table 1  
SCREENING PROTOCOL

5	Day 1	Trypsinize xpon ntially gr wing LVIP2.OZc cells, s d 5x10 <sup>5</sup> cells/10 cm- plate, incubate overnight in 10ml growth medium.
10	Day 2	Transfect cells with 10-20µg plasmid DNA using Ca,PO <sub>4</sub> technique, see Materials and Methods, incubate 15 to 24 hours at 35°C under 2-4% CO <sub>2</sub> .
15	Day 3	Wash cells 2x with growth medium, refeed, incubate 24 hours at 37°C under 5% CO <sub>2</sub> .
20	Day 4	Trypsinize cells, pellet, seed in 96 well microtiter plates 5-10x10 <sup>4</sup> cells/well, incubate overnight in 100µl growth medium.
25	Day 5	To control wells (e.g, quadruplicates) add 100µl medium + 1mM IBMX with (control stimulation) or without (control basal) 1µM forskolin. To remaining wells add agonist(s) in 100µl medium + 1mM IBMX with (G <sub>i</sub> -coupled), or without (G <sub>i</sub> -coupled) 1µM forskolin. Include antioxidants and/or protease inhibitors as required. Incubate 6-8 hours at 37°C under 5% CO <sub>2</sub> . Wash cells with 200µl PBS and drain. (Cells can be stored at -20°C for 24-72 hours).
30	Day 6	Add 25µl diluted assay buffer/well, wait 10 minutes, then add 100µl assay buffer, wait 10 minutes, then add 25µl of substrate. (ONPG 4mg/ml assay buffer). View color development visually or measure spectrophotometrically at 405nm in a plate reader.
35		

\* \* \* \*

All publications mentioned hereinabove  
are hereby incorporated in their entirety by  
reference.

5           While the foregoing invention has been  
described in some detail for purposes of clarity  
and understanding, it will be appreciated by one  
skilled in the art from a reading of this  
disclosure that various changes in form and detail  
10       can be made without departing from the true scope  
of the invention and appended claims.

**WHAT IS CLAIMED IS:**

1. A method of identifying ligands which bind to G-protein coupled receptors comprising:  
expressing a G-coupled receptor gene in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;  
adding a ligand to said cell; and  
assaying for the amount of cyclic AMP.
2. The method according to claim 1, wherein said cyclic AMP sensitive reporter construct comprises a cyclic AMP responsive enhancer element.
3. The method according to claim 2, wherein said cyclic AMP sensitive reporter construct further comprises a Lac Z gene.
4. The method according to claim 3, wherein said cyclic AMP sensitive reporter construct is pLVIP2.OZ.
5. The method according to claim 4, wherein said cell comprises a cell from cell line LVIP2.OZc.
6. The method according to claim 1, wherein said assaying step comprises adding a chromogenic substrate and assaying for change in chromogenic substrate.
7. The method according to claim 6, wherein said chromogenic substrate is o-nitrophenyl  $\beta$ -D-galactopyranoside.

8. The method according to claim 1, where in said G-coupled receptor gene is encoded by a cDNA.

9. A method of identifying an antagonist of a ligand where in said ligand binds to a G-protein coupled receptor comprising:

expressing a G-coupled receptor gene or cDNA in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding a ligand and antagonist to said cell; and

assaying for the amount of cyclic AMP.

10. The method according to claim 9, wherein said cyclic AMP sensitive reporter construct comprises a cyclic AMP responsive enhancer element.

11. The method according to claim 9, wherein said cyclic AMP sensitive reporter construct further comprises a Lac Z gene.

12. The method according to claim 11, wherein said cyclic AMP sensitive reporter construct is pLVIP2.OZ.

13. The method according to claim 12, wherein said cell comprises a cell from cell line LVIP2.OZc.

14. The method according to claim 9, wherein said assaying step comprises adding a chromogenic substrate and assaying for change in chromogenic substrate.



15. The method according to claim 14, where in said chromogenic substrate is o-nitrophenyl 8-D-galactopyranoside.

16. The method according to claim 9, wherein said G-coupled receptor gene is a G<sub>i</sub>-coupled receptor gene.

17. A method of identifying ligands which bind to a G-protein coupled receptor comprising:  
expressing a G<sub>i</sub>-coupled receptor gene of cDNA in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding forskolin and a ligand;

assaying for the amount of cyclic AMP;

and

identifying agonist induced reduction of forskolin activity.

18. A method of identifying an antagonist of a ligand which binds to a G-protein coupled receptor comprising:

expressing a G<sub>i</sub>-coupled receptor gene of cDNA in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding forskolin, ligand, and antagonist;

assaying for the amount of cyclic AMP;

and

identifying agonist induced reduction of forskolin activity.

19. A mouse L cell that comprises a recombinant cyclic AMP sensitive reporter construct.

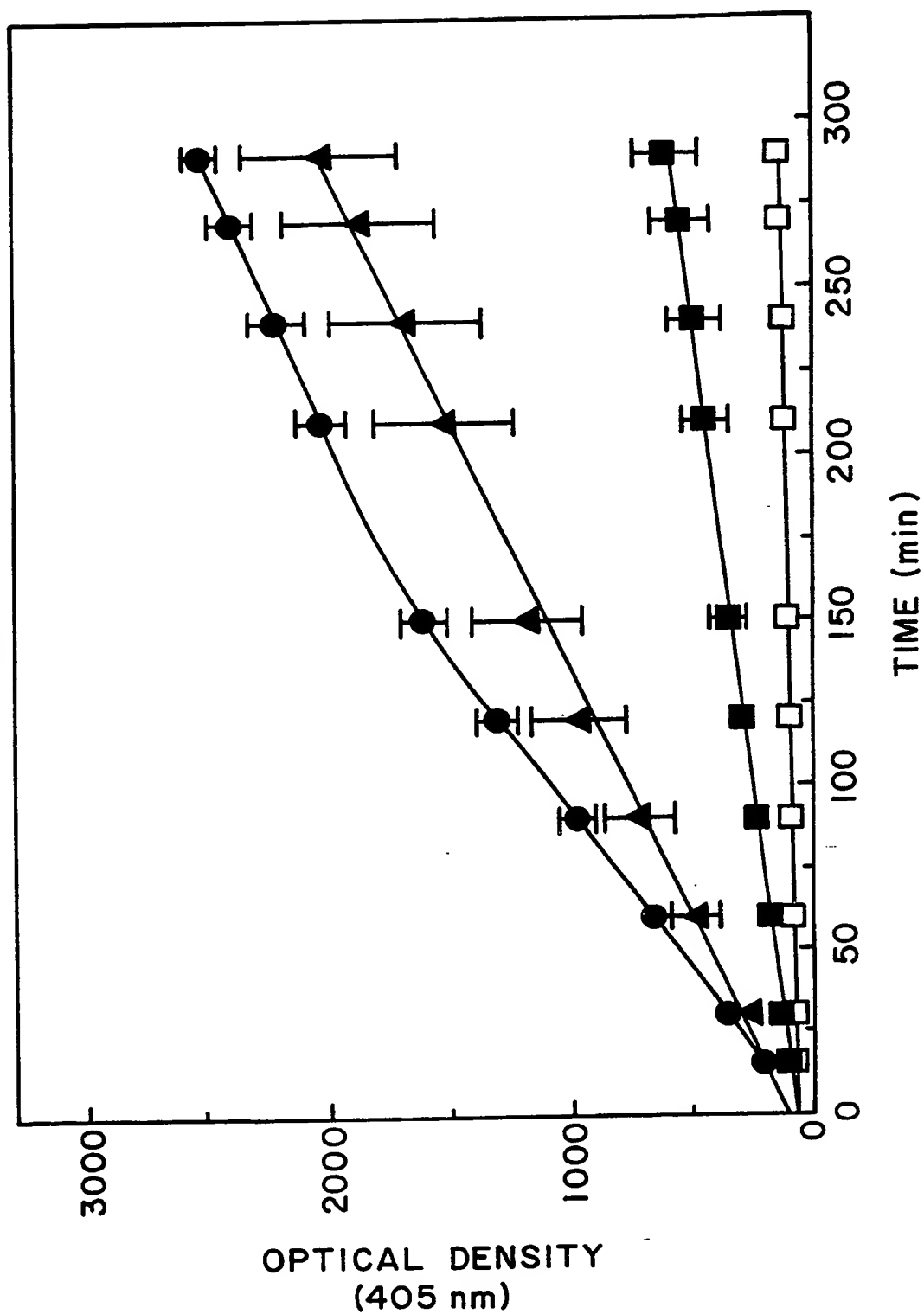
20. The cell according to claim 19, wherein said construct comprises a cyclic AMP responsive element.

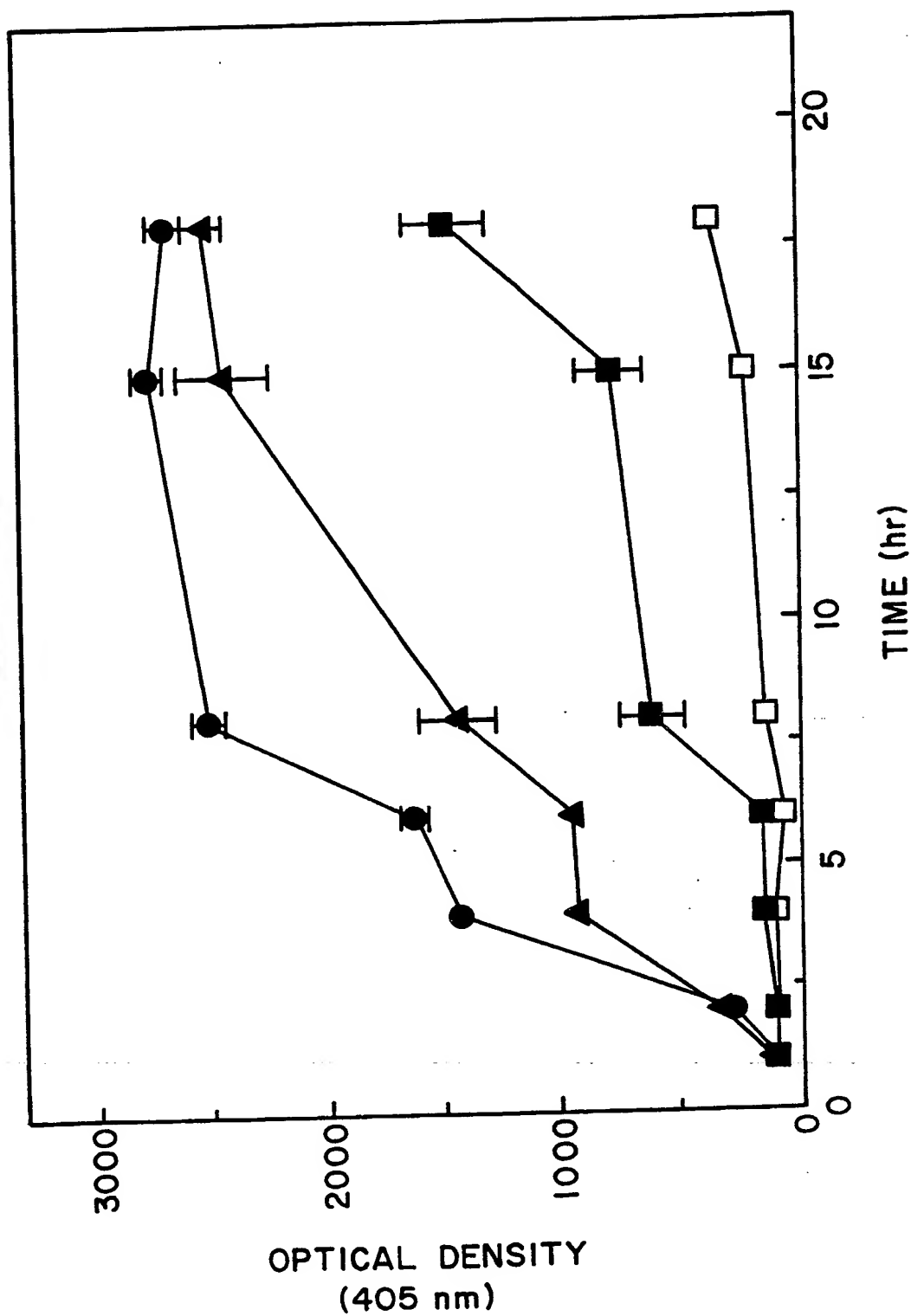
21. The cell according to claim 19, wherein said cyclic AMP sensitive reporter construct comprises a Lac Z gene.

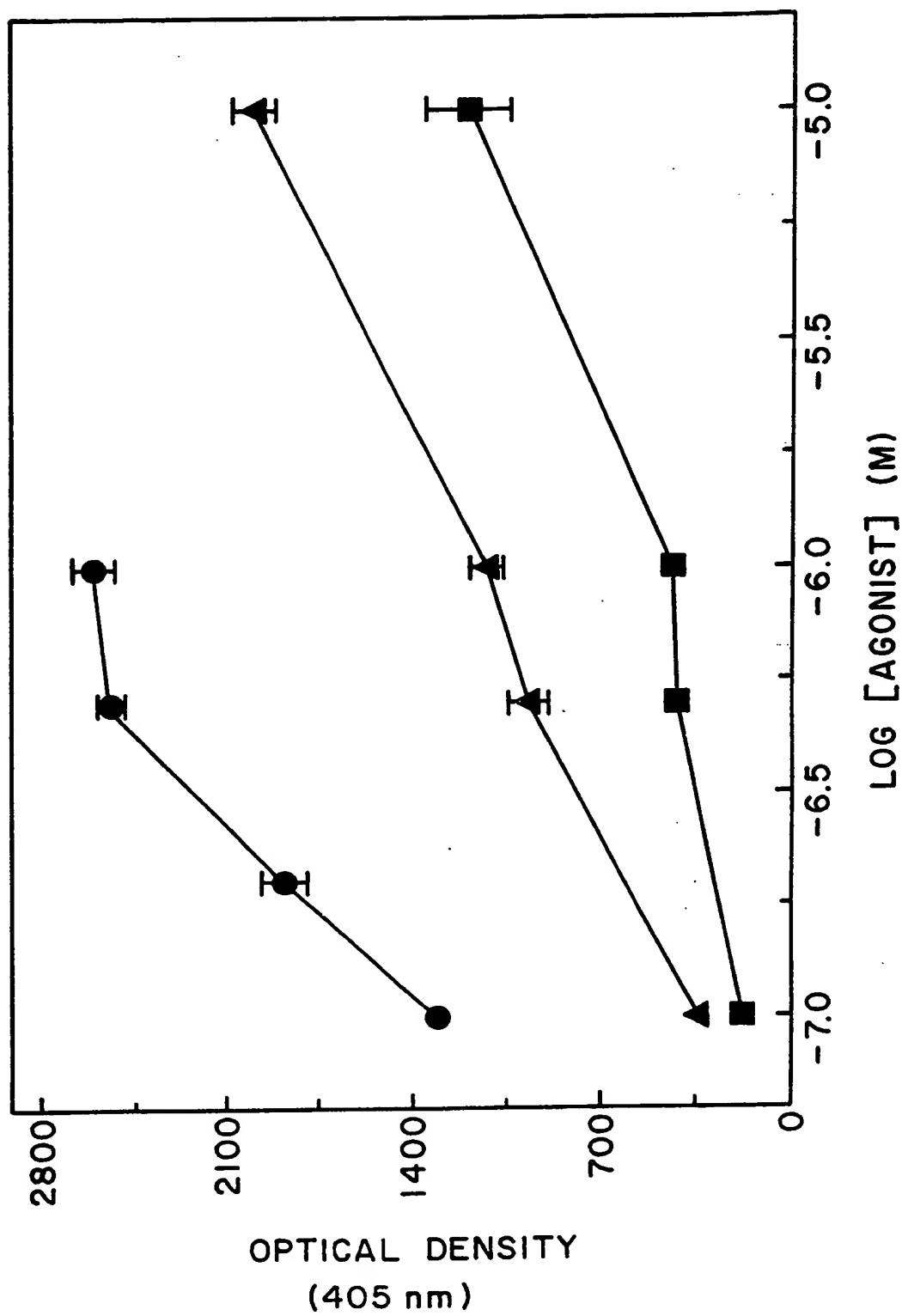
22. The cell according to claim 19, wherein said construct is stably integrated into genomic DNA of said cell.

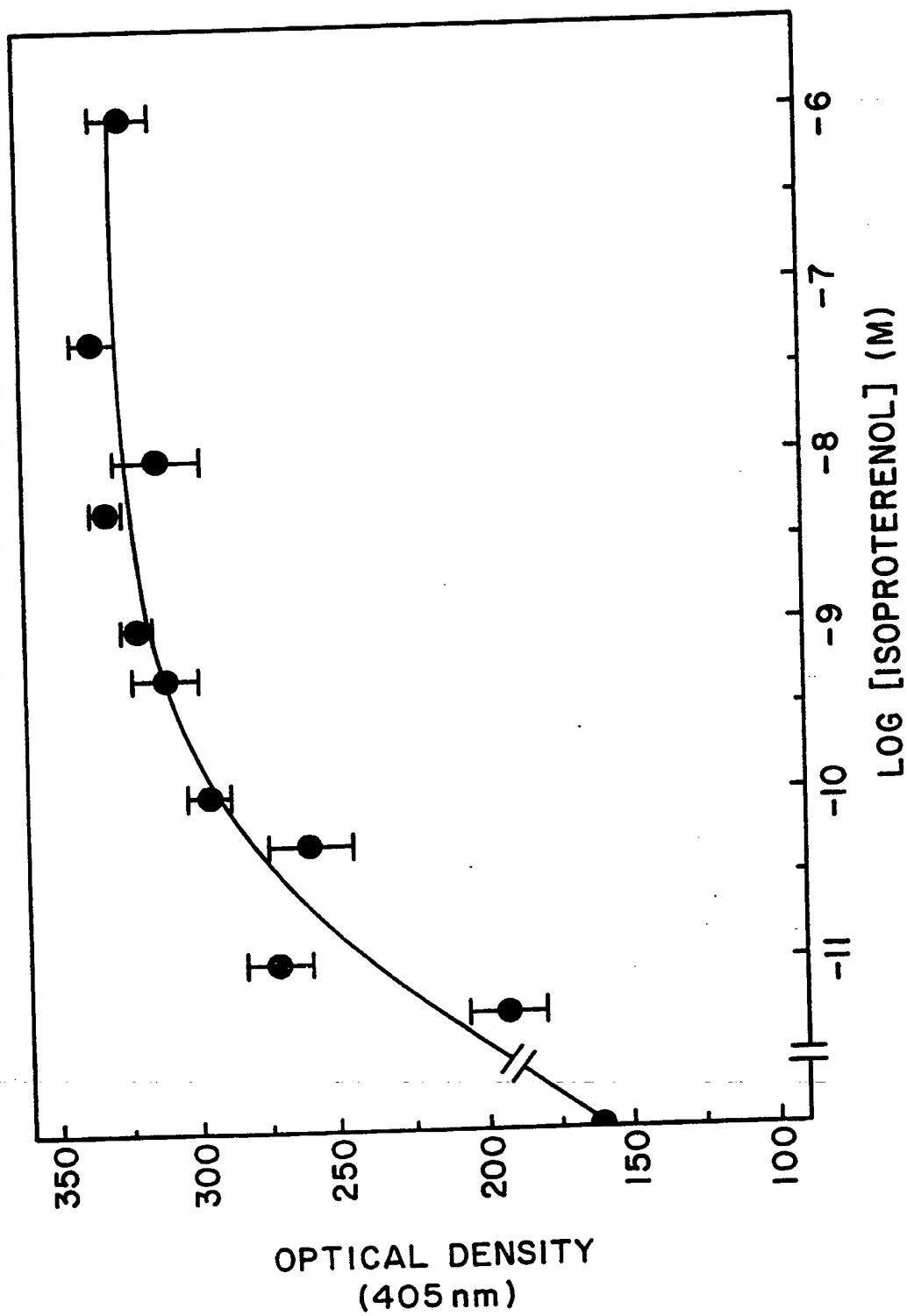
23. The cell according to claim 19, wherein said construct is pLVIP2.OZ.

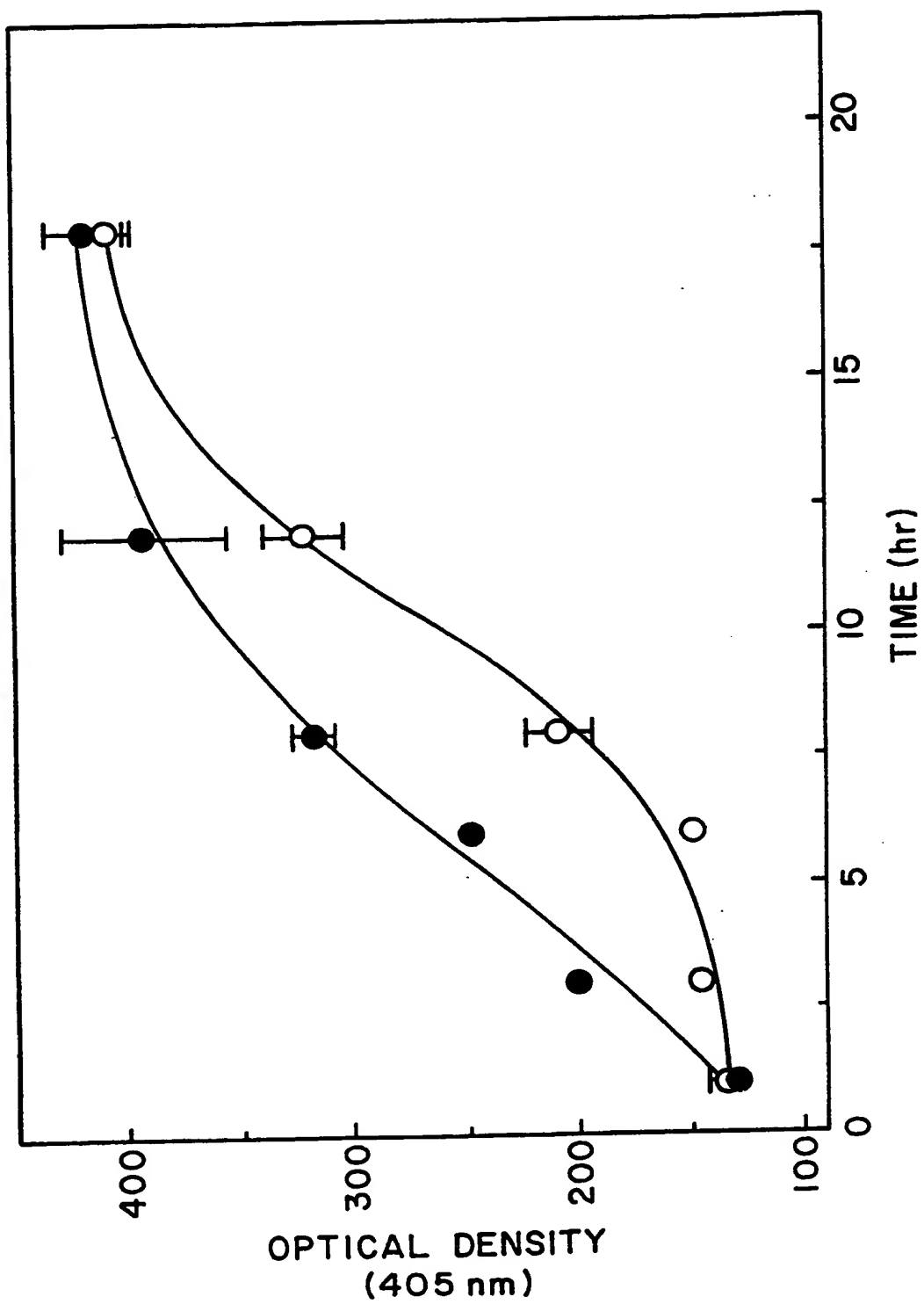
24. The cell according to claim 23, wherein said cell is a LVIP2.OZc cell.

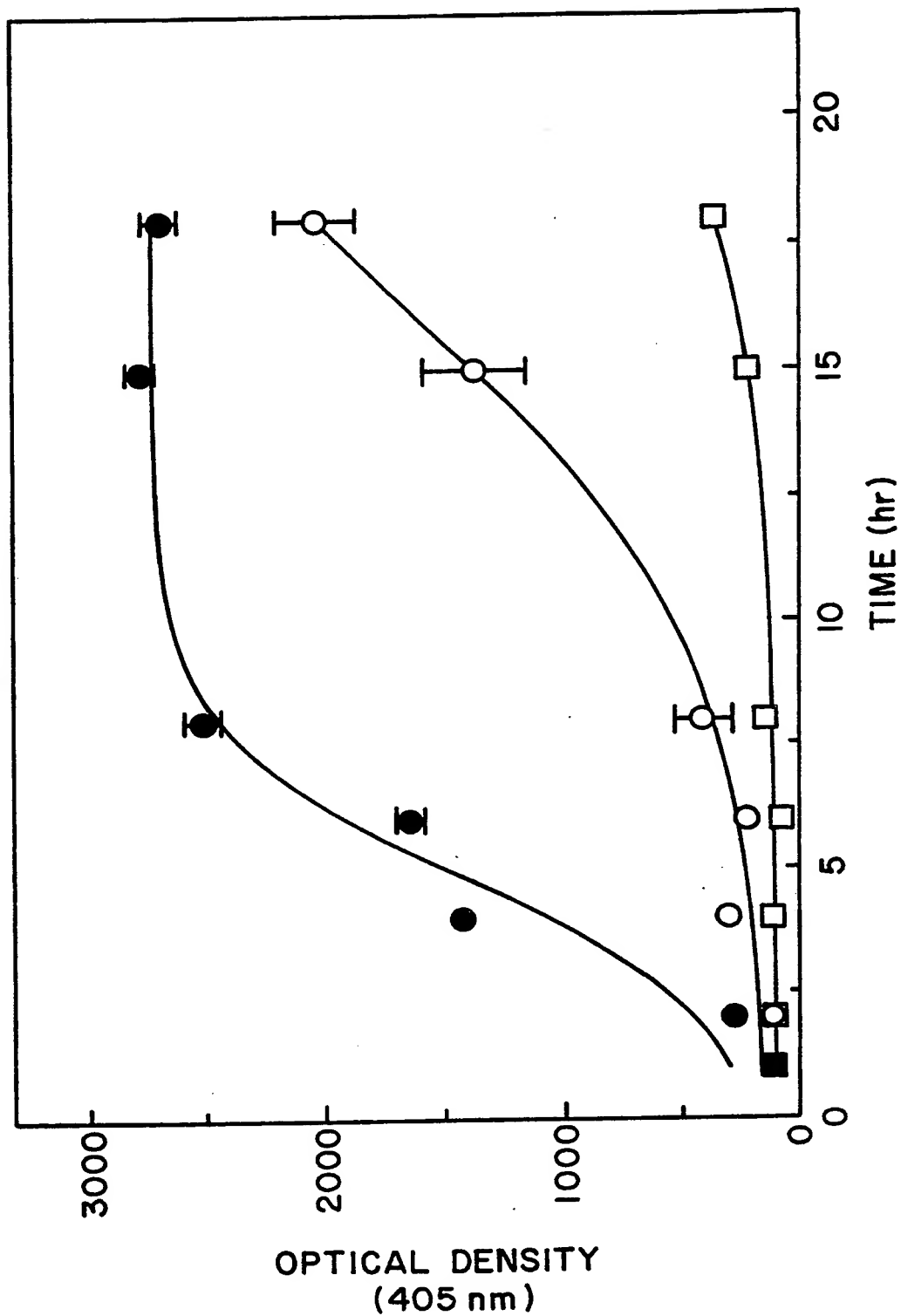
**FIG. 1**

**FIG. 2**

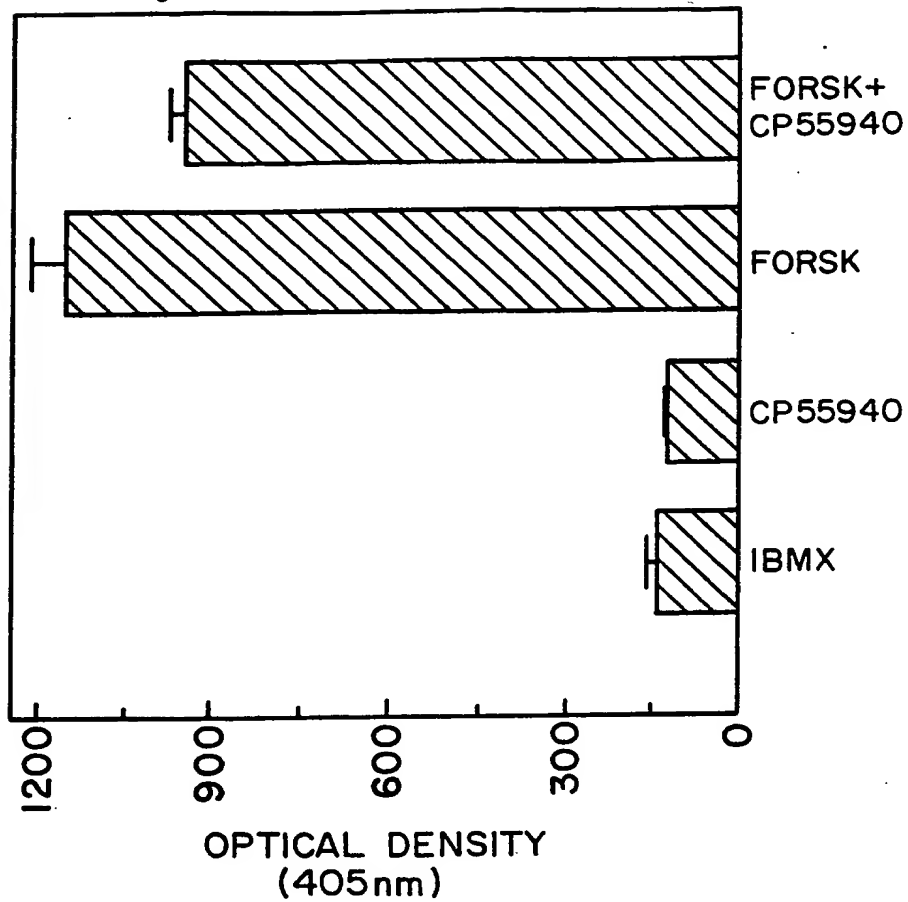
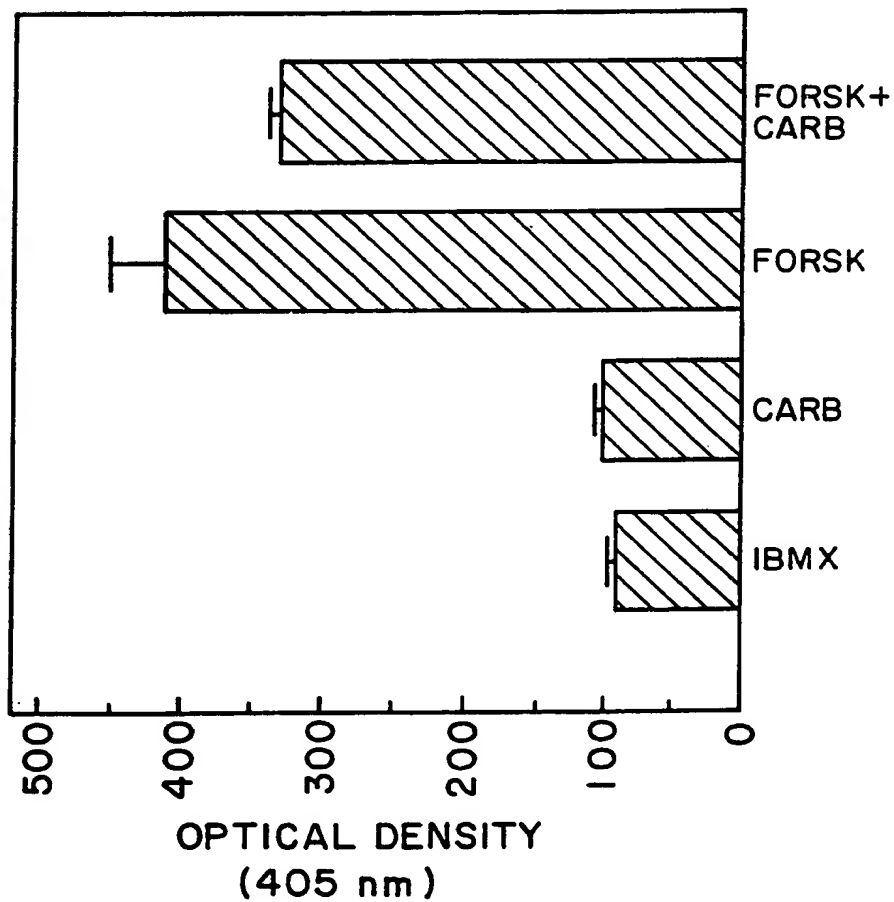
**FIG. 3**

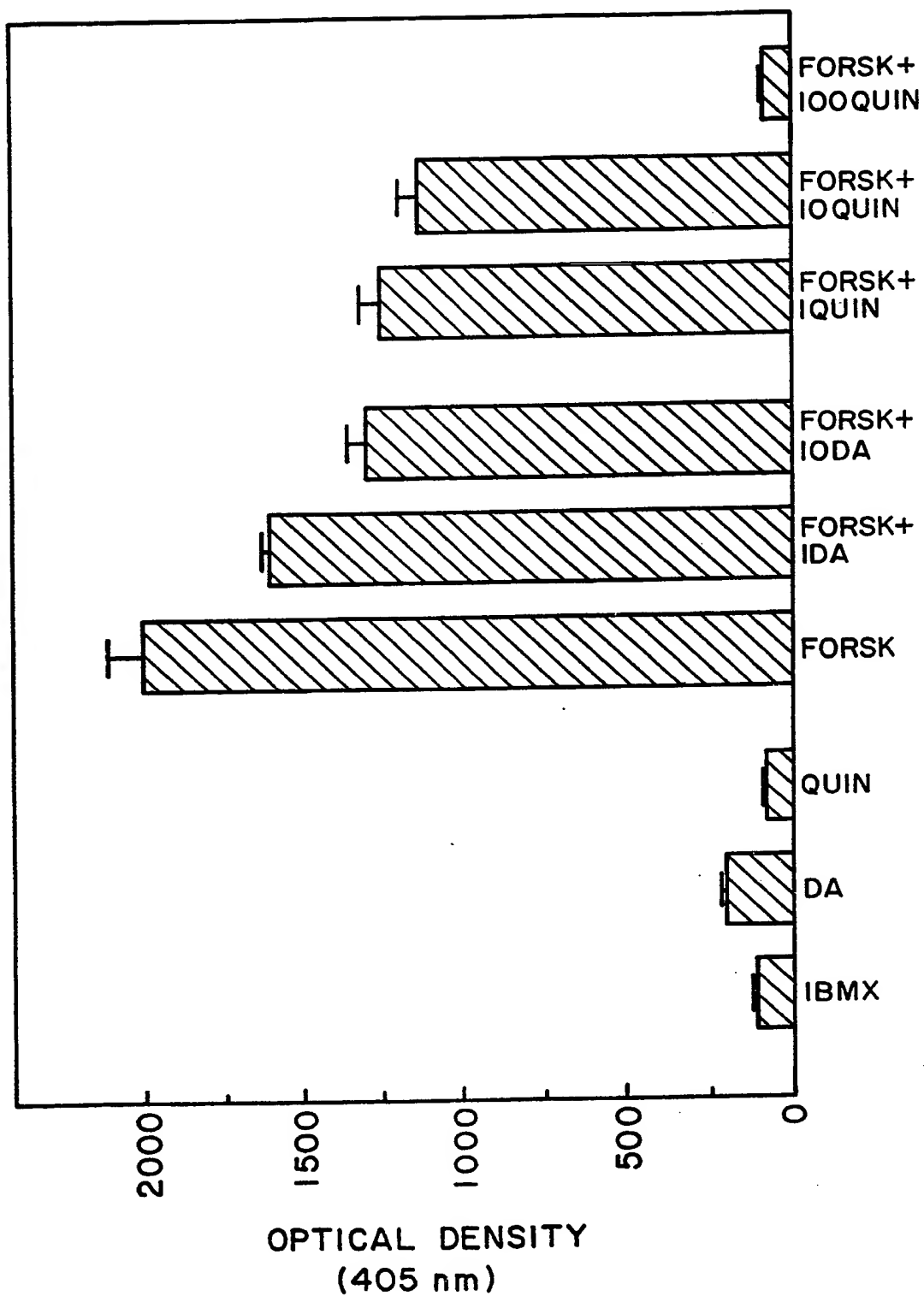
**FIG. 4a**

**FIG. 4b**

**FIG. 5**



**FIG. 6b****FIG. 6a**

**FIG. 6C**

## INTERNATIONAL SEARCH REPORT

International application N .

T/US92/08202

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C12Q 1/68, 1/00; G01N 33/53; C12P 21/06; C12N 5/00

US CL :435/6, 7.1, 7.23, 69.1, 240.1, 240.2, 240.27; 935/34, 60, 66, 70

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.23, 69.1, 240.1, 240.2, 240.27; 935/34, 60, 66, 70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, VOLUME 336, ISSUED 03 NOVEMBER 1988, RIABOWOL, "THE CATALYTIC SUBUNIT OF CAMP-DEPENDENT PROTEIN KINASE INDUCES EXPRESSION OF GENES CONTAINING CAMP-RESPONSIVE ENHANCER ELEMENTS", PAGES 83-86, ENTIRE DOCUMENT.	1-24
Y,P	US, A, 5,126,251 (MOSS ET AL) 30 JUNE 1992, CLAIMS 1-13, ENTIRE DOCUMENT.	1-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be part of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 OCTOBER 1992

Date of mailing of the international search report

08 JAN 1993

Name and mailing address of the ISA/  
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Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

GIAN WANG

Telephone N . (703) 308-0196

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